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CCK₂ receptor splice variant with intron 4 retention in human gastrointestinal and lung tumors

Meike Körner^{1,*}, Beatrice Waser², Jean Claude Reubi², and Laurence J. Miller^{1,*}

¹Mayo Clinic, Cancer Center and Department of Molecular Pharmacology and Experimental Therapeutics, Scottsdale, AZ ²Institute of Pathology of the University of Berne, Division of Cell Biology and Experimental Cancer Research, Berne, Switzerland

Abstract

The wild-type CCK₂ receptor is expressed in many gastrointestinal and lung tumors. A splice variant of the CCK₂ receptor with retention of intron 4 (CCK₂Ri4sv) showing constitutive activity associated with increased tumor growth was described in few colorectal, pancreatic, and gastric cancers. Given the potential functional and clinical importance of this spliceoform, its occurrence was quantitatively characterized in a broad collection of 81 gastrointestinal and lung tumors, including insulinomas, ileal carcinoids, gastrointestinal stromal tumors (GIST), gastric, colorectal, and pancreatic ductal adenocarcinomas, cholangiocellular and hepatocellular carcinomas, small cell lung cancers (SCLC), non-SCLC, and bronchopulmonary carcinoids, as well as 21 samples of corresponding normal tissues. These samples were assessed for transcript expression of total CCK₂ receptor, wild-type CCK₂ receptor, and CCK₂Ri4sv with end-point and real-time RT-PCR, and for total CCK₂ receptor protein expression on the basis of receptor binding with in vitro receptor autoradiography. Wild-type CCK₂ receptor transcripts were found in the vast majority of tumors and normal tissues. CCK₂Ri4sv mRNA expression was present predominantly in insulinomas (incidence 100%), GIST (100%), and SCLC (67%), but rarely in pancreatic, colorectal, and gastric carcinomas and non-SCLC. It was not found in wild-type CCK₂ receptor negative tumors or any normal tissues tested. CCK₂Ri4sv transcript levels in individual tumors were low, ranging from 0.02% to 0.14% of total CCK₂ receptor transcripts. In conclusion, the CCK₂Ri4sv is a marker of specific gastrointestinal and lung tumors. With its high selectivity for and high incidence in SCLC and GIST, it may represent an attractive clinical target.

Keywords

CCK₂ receptor intron 4 splice variant; RT-PCR; receptor autoradiography; gastrointestinal stromal tumor; small cell lung cancer

Introduction

The cholecystokinin type 2 (CCK₂) receptor is a G protein-coupled, 7 transmembrane domain receptor. Physiologically, it is expressed mainly in the brain and gastrointestinal tract where it mediates the effects of gastrin on gastric acid secretion and epithelial and endocrine cell growth [1]. The CCK₂ receptor is also a cancer biomarker of increasing clinical importance. It is expressed by a wide variety of tumors in and outside the

*Correspondence and reprint requests to: Meike Körner, M.D., Institute of Pathology of the University of Berne, Murtenstrasse 31, CH – 3010 Berne, Switzerland; Tel.: +41 31 632 3243, Fax: +41 31 632 8999, meike.koerner@pathology.unibe.ch or Laurence J. Miller, M.D., Mayo Clinic, 13400 East Shea Blvd, Scottsdale, AZ 85259; Tel.: 480 301 6650, Fax: 480 301 6969, ljm@mayo.edu.

gastrointestinal tract. It is present at especially high levels in medullary thyroid carcinomas, small cell lung cancer (SCLC), gastrointestinal stromal tumors (GIST), and insulinomas [2]. This has led to the successful development of clinical applications using radioactive CCK₂ receptor ligands for imaging and targeted radiotherapy of some of these tumors [3]. Conversely, the effects of the CCK₂ receptor on proliferation particularly of gastric and pancreatic cancer could, unfortunately, not yet be translated into established therapeutic strategies [4,5].

Tumoral CCK₂ receptors have been found to correspond not only to the wild-type form. A CCK₂ receptor splice variant that has the fourth intron inappropriately retained (CCK₂Ri4sv) has been identified in cancer [6,7]. The molecular basis for this missplicing event was shown to be a weak 3' splice site of intron 4 of the CCK₂ receptor in association with reduced splice factor levels [7]. Intron 4 retention leads to insertion of an additional 69 amino acids in the third intracellular loop of the CCK₂ receptor protein, a domain important for signal transduction. Correspondingly, the splice variant was found to differ from the wild-type CCK₂ receptor with respect to activation of intracellular signaling pathways, while ligand binding characteristics were unchanged [8]: In specific cell types, the CCK₂Ri4sv was constitutively active, leading to increased basal calcium mobilization and MAP kinase and Src kinase phosphorylation as compared with cells expressing wild-type CCK₂ receptor [6,9,10]. Expression of constitutively active CCK₂Ri4sv was associated with increased basal cell proliferation, and activation of the CCK₂Ri4sv by ligand binding resulted in enhanced cell growth compared with the wild-type receptor [6,8]. A second aberrant CCK₂ receptor form that was identified in cancer, namely in a single case of human colorectal carcinoma, was a mutant with a single point mutation in the third intracellular loop [11]. This receptor form also led to increased cell proliferation, was, however, not associated with a tumorigenic potential as observed for experimental, not naturally occurring CCK₂ receptor mutants [12].

Splice variants with specific expression and functions in cancer such as the CCK₂Ri4sv have been receiving increasing attention because of their possible role in tumor biology and potential use for diagnostic and/or therapeutic applications [13,14]. One important step toward a better understanding of their functional or clinical relevance in cancer is the validation of their expression in human tumor tissues with respect to for instance their tumor selectivity, frequency, and quantity [15]. As for the CCK₂Ri4sv, only selected tumors have been investigated for this missplicing event so far. While it has been found in colorectal, pancreatic, and gastric adenocarcinomas as well as in Barrett's mucosa, a cancer precursor lesion [6,7,16–18], it has not been quantified in all of these tissues. Moreover, tumors known for their particularly high CCK₂ receptor levels have not been considered for their CCK₂Ri4sv expression. In fact, the molecular mechanisms leading to intron 4 retention may not be restricted to the tumor types investigated so far, but could occur universally in cancers expressing the CCK₂ receptor.

Therefore, the aim of the present study was to quantitatively characterize the total CCK₂ receptor, wild-type CCK₂ receptor, and CCK₂Ri4sv expression at the transcriptional and protein level in a broader spectrum of different tumor types using end-point and real-time reverse transcription-polymerase chain reaction (RT-PCR) and in vitro receptor autoradiography. It was furthermore intended to assess the cancer selectivity of the CCK₂Ri4sv by including normal tissues of tumor origin in the study. The primary focus was laid on gastrointestinal and lung tumors, as the CCK₂ receptor is a marker for the major tumors of these organ systems [2].

Materials and Methods

Tissues

Human tumor and normal tissue samples were obtained from surgical resection specimens, immediately frozen, and stored at -80°C . A total of 81 tumor samples was investigated, including 4 insulinomas, 8 ileal carcinoid tumors, 4 GIST, 8 gastric adenocarcinomas, 13 colorectal adenocarcinomas, 7 pancreatic ductal adenocarcinomas, 3 cholangiocellular carcinomas, 5 hepatocellular carcinomas, 9 SCLC, 12 non-SCLC (nSCLC), and 8 bronchopulmonary carcinoid tumors. Furthermore, 3 samples of each normal gastric mucosa, gastric muscularis layer, ileal mucosa, colorectal mucosa, pancreas, liver, and lung were assessed. The tissues originated either from samples investigated previously for peptide receptors [19–25] collected in accordance with the required international ethical guidelines or from samples collected prospectively at the Institute of Pathology of the University of Berne in agreement with the World Medical Association Declaration of Helsinki, including informed consent and approval by the Institutional Review Board.

In vitro receptor autoradiography for binding sites of CCK₂ receptor protein

In vitro receptor autoradiography to assess the expression of CCK₂ receptor binding sites in tumor tissues was performed as described previously [19]. Briefly, 20 μm -thick tissue sections mounted on glass slides were incubated with 2,000 Ci/mmol of the radioligand [¹²⁵I]-D-Tyr-Gly-[(Nle^{28,31})CCK-26–33] (¹²⁵I-CCK; Anawa, Wangen, Switzerland) either alone or in competition with 50 nM cold sulfated CCK-8 (Bachem, Bubendorf, Switzerland) or 50 nM cold gastrin (Bachem). Thus, CCK₂ receptors can be distinguished from CCK₁ receptors, as the former show a high affinity for both CCK-8 and gastrin, whereas the latter bind only CCK-8 with high affinity. The slides were then exposed to a radiation-sensitive film. The density of the CCK₂ receptor-specific signal on the film was quantified in the tumor region that was later excised from the original tumor sample for PCR analysis. This was achieved with a computer-assisted image processing system (Interfocus, Mering, Germany) and radioactive tissue standards (Autoradiographic [¹⁴C] microscales, GE Healthcare, Little Chalfont, UK) containing known amounts of isotope, cross-calibrated to tissue-equivalent ligand concentrations [26,27].

RNA extraction and reverse transcription

A tumor region either positive or negative for CCK₂ receptor binding sites by autoradiography was selected and cut out of the frozen tissue block with a sterile scalpel blade. Care was taken to choose an area of tumor tissue without admixed normal tissue with the help of a matching H&E-stained tissue section. Similarly, aliquots of normal tissues were obtained. The dissected tissue samples (≤ 5 mg) were disrupted and homogenized using the TissueLyser (Qiagen, Hilden, Germany), and total RNA was isolated with the RNeasy Micro Kit (Qiagen), with inclusion of a DNase I digestion step. The total RNA concentration was measured by spectrophotometry, and the RNA quality was checked with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Reverse transcription to cDNA was performed with SuperScript III (Invitrogen, Carlsbad, CA) using oligo(dT)s or random hexamers. For end-point PCR, 500 ng total RNA was converted per 20 μl -volume reaction.

End-point PCR for wild-type CCK₂ receptor and CCK₂Ri4sv transcripts

CCK₂ receptor transcripts were identified with a nested PCR, where the first round reaction amplified total CCK₂ receptor cDNA, and the second round reaction either wild-type CCK₂ receptor or CCK₂Ri4sv. The primer characteristics are shown in table 1. PCR was carried out on 2 μl of cDNA or first round PCR product with 5 U/ml Platinum Taq Polymerase

(Invitrogen), 2 mM MgCl₂ (Invitrogen), 0.2 mM dNTPs (Stratagene, La Jolla, CA), and 0.2 μM of both sense and antisense primers in a 50 μl reaction volume. The first round reaction was cycled 35 times for 30 sec at 94°C, 45 sec at 57°C, and 3 min at 72°C. The second round reaction included 37 cycles of 30 sec at 94°C, 45 sec at 57°C for the wild-type receptor or 50 sec at 55°C for the splice variant, and 45 sec at 72°C. Reactions were run at least in duplicate. As positive controls, the previously described CHO cell lines stably expressing wild-type CCK₂ receptor (CHO-CCK₂R cells) or CCK₂Ri4sv (CHO-CCK₂R-i4 cells) [8] were used, and for negative control no template was added. Furthermore, β-actin control PCR reactions were run for each sample with 32 cycles comprising 30 sec at 94°C, 45 sec at 59°C, and 1 min at 72°C (table 1). Fifteen μl-fractions of final PCR products were resolved on a 1.5% agarose/TAE gel along with a 100 bp DNA Ladder (New England Biolabs, Ipswich, MA) as molecular weight marker and visualized by ethidium bromide staining. Representative bands were excised and sequenced to confirm their identities.

Real-time PCR for total CCK₂ receptor and CCK₂Ri4sv transcripts

Of 52 of the 63 tumors positive for CCK₂ receptor transcripts by end-point PCR, sufficient RNA was available for further real-time PCR analysis. Two different real-time PCR reactions were established to amplify either total CCK₂ receptor or CCK₂Ri4sv cDNA. Primers and the TaqMan MGB probe (table 1) were designed using the Primer Express software version 3.0 (Applied Biosystems, Foster City, CA) and synthesized by Applied Biosystems. The optimal primer and probe concentrations yielding the lowest Ct and highest ΔRn values for a given RNA concentration were found to be 900 nM for all primers and 250 nM for the probe. Using these concentrations, cDNA corresponding to 100ng total RNA was amplified in a 20 μl reaction volume with the TaqMan Universal PCR Master Mix (Applied Biosystems) on a 7900HT Fast Real-time PCR System (Applied Biosystems) as follows: 2 min at 50°C, 10 min at 95°C, followed by 50 cycles of 15 sec at 95°C and 1 min at 60°C. The endogenous control genes 18S rRNA, GAPDH, and human β-actin were amplified in each sample using pre-designed primer and probe sets by Applied Biosystems. Standard curves for all genes were constructed using serial cDNA dilutions of CHO-CCK₂R or CHO-CCK₂R-i4 cells or the pancreatic cancer cells CAPAN2 or BxPC3 (American Type Culture Collection, Manassas, VA). On each plate, the same CCK₂ receptor expressing tumor was included as calibrator, and no template reactions as negative controls. All reactions were run at least in triplicate.

CCK₂ receptor gene expression was relatively quantified using the efficiency (E)-corrected ΔCt method. Total CCK₂ receptor transcript levels were expressed relative to 18S rRNA and a calibrator sample using the equation:

$$E(\text{total CCK}_2\text{R})^{\Delta\text{Ct}(\text{total CCK}_2\text{R})(\text{calibrator}-\text{sample})} / E(18\text{S rRNA})^{\Delta\text{Ct}(18\text{S rRNA})(\text{calibrator}-\text{sample})} \quad [28].$$

CCK₂Ri4sv transcript expression was reported relative to total CCK₂ receptor transcript expression as follows:

$$E(\text{CCK}_2\text{Ri4sv})^{-\text{Ct}(\text{CCK}_2\text{Ri4sv})} / E(\text{total CCK}_2\text{R})^{-\text{Ct}(\text{total CCK}_2\text{R})} \quad [29, 30].$$

Immunohistochemistry for the proliferation marker MIB-1

Immunohistochemistry for the proliferation marker MIB-1 was performed on 9 SCLC, 4 nSCLC, and 4 pancreatic adenocarcinomas. Cryostat sections (10 μm thick) were postfixed in formalin. The primary antibody (DAKO, Zug, Switzerland) was added in a 1:50 dilution. The secondary antibody was a biotinylated goat anti-mouse immunoglobulin (Dako).

Antibody binding was visualized using the VECTASTAIN Elite ABC kit (Vector, Burlingame, CA). Staining was carried out with DAB, and counterstaining with hemalum.

Statistical analysis

Statistical analysis was performed with the GraphPad Prism software version 4.0 (La Jolla, CA), using the unpaired t-test, Mann-Whitney test, linear regression analysis and Spearman correlation. $P < 0.05$ was considered statistically significant.

Results

Real-time PCR: specificity, dynamic range, normalization

Several lines of evidence of the specificities of the real-time PCR reactions for total CCK₂ receptor and the CCK₂Ri4sv, respectively, were obtained. By separating amplification products on an agarose gel, each reaction yielded a single band migrating with the expected molecular size (figure 1A). Moreover, the reaction for CCK₂Ri4sv produced no amplification signal in CHO-CCK₂R cells which express only wild-type, but not splice variant CCK₂ receptor. Finally, in samples where the reverse transcriptase had been omitted, no amplification signal was obtained.

The real-time PCR assays for total and splice variant CCK₂ receptors were tested on serial cDNA dilutions of CHO-CCK₂R and CHO-CCK₂R-i4 cells, respectively. In both assays, the relationship between the Ct values, which spanned the Ct values anticipated for the samples, and the logarithm of the starting RNA amount remained linear over the tested 6-log concentration range (figure 1B). Similar plots were created for 18S rRNA, GAPDH, and β -actin, yielding slopes of -3.40 , -3.39 , and -3.50 , respectively, and $r^2 > 0.99$ for all three genes. These plots were then generated in each real-time PCR run, to calculate the efficiencies used for relative quantification.

The endogenous control genes 18S rRNA, GAPDH, and β -actin were evaluated for their suitability for normalization, while a larger number of candidate genes could not be tested due to limited availability of sample RNA. The expression levels of these genes were determined in each sample using the formula E^{-Ct} . 18S rRNA expression was fairly constant among the various tumor groups, whereas expression of GAPDH and β -actin was much more variable. We concluded that 18S rRNA was the most suitable endogenous control in the studied tumor population and, therefore, used it for relative quantification of total CCK₂ receptor transcript levels.

Wild-type CCK₂ receptor transcript expression

The wild-type CCK₂ receptor transcript expression was assessed with end-point PCR. It was found to be very common (table 2). It was detected in every tumor type studied, except for cholangiocellular carcinoma. Moreover, the frequency of the wild-type CCK₂ receptor transcript expression in the individual tumor types was mostly high. Furthermore, all investigated normal tissues were positive for the wild-type CCK₂ receptor except for the lung. Typical end-point PCR results for the wild-type CCK₂ receptor in tumors and normal tissues are shown in figure 2A in the top gel.

CCK₂Ri4sv transcript expression

Using end-point PCR, CCK₂Ri4sv transcripts were found in a subset of tumors expressing wild-type CCK₂ receptor mRNA, while they were not detected in tumors without wild-type receptors. Unlike the wild-type form, the splice variant was restricted to specific tumor types (table 2, figure 2A). It was commonly expressed in insulinomas, GIST, and SCLC. Indeed, in the former two tumor types it was present in every case studied. By contrast, the

CCK₂Ri4sv expression was rare in pancreatic adenocarcinomas and nSCLC, where only single cases were found to be positive. Splice variant transcripts were not identified in the remaining tumors. Likewise, they were not detected in any of the normal tissue samples.

In the tumors that expressed the CCK₂Ri4sv by end-point PCR, the transcript levels were quantified relative to total CCK₂ receptor levels with real-time PCR. Splice variant transcripts were found to account for a very small fraction of all CCK₂ receptor transcripts. The mean expression levels ranged from 0.21‰ of total CCK₂ receptors in GIST to 1.05‰ in SCLC (table 2). The expression levels in the individual cases are shown in figure 3A. This figure also demonstrates how the relative splice variant amounts varied between the different tumor types. In SCLC, a significantly higher proportion of all CCK₂ receptor transcripts corresponded to the splice variant than in GIST ($p=0.0003$) or insulinomas ($p=0.0045$).

Real-time PCR analysis furthermore revealed that the identification of the CCK₂Ri4sv was associated with the amount of total CCK₂ receptor mRNA. Tumors with splice variant expression showed significantly higher total receptor transcript levels than tumors without ($p<0.0001$). This is shown in figure 3B for the individual cases. The splice variant was identified only in tumors with total CCK₂ receptor mRNA levels above a certain level. Conversely, in only two of the 37 tumors without splice variant expression, the total CCK₂ receptor mRNA amounts equaled those of tumors expressing the splice variant, while the remaining tumors showed lower total receptor transcripts levels.

Immunohistochemistry for the proliferation marker MIB-1 was performed in selected tumor samples to assess the relation between CCK₂Ri4sv expression and the proliferative index. In SCLC, the mean proportion of tumor cells in the proliferative phase, as visualized by nuclear staining for MIB-1, was larger in tumors with than in tumors without CCK₂Ri4sv expression (64.8% MIB-1-positive tumor cells in SCLC with CCK₂Ri4sv expression vs. 56.5% in SCLC without CCK₂Ri4sv expression). This difference was, however, not statistically significant ($p=0.2774$). Conversely, in nSCLC and pancreatic adenocarcinomas, the single cases expressing CCK₂Ri4sv exhibited a lower proliferative index than the tumors without splice variant expression (nSCLC: 30.5% vs. mean 37.7% MIB-1-positive tumor cells; pancreatic adenocarcinomas: 9% vs. mean 30.1%).

Total CCK₂ receptor binding site expression

Total CCK₂ receptor protein expression in tumor tissues was assessed on the basis of CCK₂ receptor binding with in vitro receptor autoradiography [19]. With this method, the binding sites of both wild-type CCK₂ receptors and CCK₂Ri4sv are simultaneously identified, as these two receptor forms cannot pharmacologically be differentiated [8]. The autoradiography results were comparable with published data (table 2) [2]: The CCK₂ receptor binding site expression with respect to both incidence and density was particularly high in insulinomas, GIST, and SCLC, but virtually absent in gastric, colorectal, and pancreatic carcinomas, liver tumors, and nSCLC. This is illustrated with two representative cases in figure 2B. The GIST in the first row displays strong ¹²⁵I-CCK binding, corresponding to a high number of CCK receptor binding sites; displacement of ¹²⁵I-CCK by both CCK-8 and gastrin provides proof of specific CCK₂ receptor identification. Conversely, in the colorectal carcinoma in the second row there are no CCK receptor binding sites identifiable, as this tumor shows no specific ¹²⁵I-CCK binding.

Correlation of CCK₂ receptor binding site and transcript expression in tumor tissues

Receptor binding sites were not identified in over half of the tumors showing CCK₂ receptor transcripts (table 2). Real-time PCR analysis revealed that these autoradiographically

negative tumors showed particularly low total CCK₂ receptor transcript levels, while tumors with detectable receptor binding sites displayed significantly higher receptor mRNA amounts ($p<0.0001$). This is demonstrated for the individual tumors in figure 3B. Furthermore, it was found that in autoradiographically positive tumors the actual binding site density deviated considerably from that predicted from the transcript levels in a linear regression model in the majority of cases, as can be seen in figures 4A and B. Taking into account all autoradiographically positive and negative cases, the density levels of CCK₂ receptor binding sites correlated statistically fairly well with total CCK₂ receptor transcript levels ($r^2=0.6579$, $p=0.0002$).

Discussion

The CCK₂ receptor splice variant with intron 4 retention was previously described in a limited number of colorectal, pancreatic, and gastric adenocarcinomas [6,7,16,17]. It was considered to be important in these particular tumors, due to its special functional characteristics, namely constitutive activity associated with increased proliferation [6]. By quantitatively assessing the CCK₂Ri4sv expression in a larger number of gastrointestinal and lung tumors, the present study reveals that this splice variant is in fact a marker of a different spectrum of tumors. It was found in several tumor types that up to now have not been considered for this splicing event, including such diverse tumors as insulinomas, GIST, SCLC, and nSCLC. In most of these tumors the CCK₂Ri4sv was expressed in high frequency, whereas it was rare or not detected in colorectal, pancreatic, and gastric adenocarcinomas. The CCK₂Ri4sv was never the only CCK₂ receptor form present in the tumors. Indeed, it accounted for only a small fraction of all CCK₂ receptor transcripts. The occurrence of the CCK₂Ri4sv was strongly associated with elevated total CCK₂ receptor mRNA levels. This is suggestive of an intrinsic small likelihood of intron 4 not to be recognized by the splicing machinery due to its weak 3' splice site [7] which reaches detection levels when enough CCK₂ receptor is transcribed. Conversely, the significant differences in the relative CCK₂Ri4sv expression levels observed between the various tumor types indicate additional tumor-specific aberrant molecular processes involved in missplicing of the CCK₂ receptor. The tumoral CCK₂Ri4sv expression was not significantly associated with an increased proliferation rate.

Existing data indicate a high selectivity of the CCK₂Ri4sv for tumors over non-neoplastic tissues, which is in fact much higher than that of the wild-type CCK₂ receptor [6,7,16,17,31]. In the present series, the CCK₂Ri4sv was not identified in a variety of normal gastrointestinal and lung tissues. It was in particular not detected in normal tissues of origin of tumors that frequently express it, such as gastric muscularis and lung. This extends previous findings of the absence of CCK₂Ri4sv in normal colon and pancreas [6,7,16]. Of note, the CCK₂Ri4sv may not absolutely be restricted to neoplasia, as it has been described in peripheral blood mononuclear cells of a minority of healthy individuals and in normal gastric mucosa [17,31].

For some tumor and normal tissues, discrepancies exist between various reports concerning the presence of CCK₂Ri4sv mRNA. Colorectal cancer was originally reported to express CCK₂Ri4sv, but not wild-type CCK₂ receptor transcripts [6]. However, Schmitz et al. recognized only the wild-type, but not the splice variant receptor in as many as 79 colorectal carcinomas [11], in full agreement with our present findings. Furthermore, we could not detect the CCK₂Ri4sv in gastric cancer or normal gastric mucosa where it had been described before [17]. Finally, the single cases of CCK₂Ri4sv-expressing pancreatic adenocarcinomas reported in the literature suggested frequent CCK₂ receptor missplicing in these tumors [7,16]. However, in this study the splice variant could be identified in only one out of eight investigated cases. This variable identification of CCK₂Ri4sv transcripts may at

least in part be due to the very low expression levels. The amount of RNA employed for RT-PCR could become critical. Zhou et al. used ten times more RNA than we were able to utilize [17]. Furthermore, primers may underperform in amplification of low abundant transcripts when located in the vicinity of AG-rich stretches [32] which are present in intron 4 of the CCK₂ receptor. On the other hand, it has to be considered that primers not able to distinguish between amplified cDNA and genomic DNA [6,16] may be responsible for a false high incidence of splice variants with intron retention.

Divergent data has also accumulated over the last years on the CCK₂ receptor protein and transcript expression in gastrointestinal tumors, in particular in colorectal, pancreatic, and gastric adenocarcinomas [5]. In these tumors, the CCK₂ receptor incidence was considerably lower when assessed with receptor binding studies compared with RT-PCR. In the present study, both methods were for the first time applied in parallel on the same tumor specimens, and the quantitative expression of total tumoral CCK₂ receptor binding sites was correlated with that of total CCK₂ receptor mRNA. This revealed that the cases with divergent results, i.e. negative autoradiography but positive RT-PCR, consistently expressed very low amounts of total CCK₂ receptor transcripts. This may reflect a lower sensitivity of CCK₂ receptor autoradiography compared with RT-PCR. The correlation furthermore demonstrated that CCK₂ receptor transcript levels could not reliably predict the amounts of CCK₂ receptor binding sites in a substantial number of cases. Possible explanations for this non-linear relationship between CCK₂ receptor mRNA and protein levels are for instance variations in the protein translation rate or in the compartmentalization of the receptor protein in intracellular pools [33] between individual tumors.

The protein expression of the CCK₂Ri4sv cannot specifically be assessed in tissues, as selective ligands or immunohistochemical antibodies are not available. In transfected cell lines, it could, however, be shown that the CCK₂Ri4sv translated into protein to a similar extent as the wild-type receptor [8]. If this also holds true for original tumors can only be speculated at present. In this case, the density levels of total CCK₂ receptor binding sites, which most probably correspond largely to wild-type receptors, would suggest very low CCK₂Ri4sv protein amounts in tumors. It has to be considered, though, that the splice variant protein may not show the same stability or be expressed to the same extent in the plasma membrane as the wild-type receptor. Indeed, in transfected cell lines the CCK₂Ri4sv was found to be located mainly in the intracellular compartment, whereas the wild-type receptor was expressed predominantly in the cell membrane [34].

The CCK₂Ri4sv has been shown to stimulate cell proliferation in vitro [6,7,9,35]. In the present study, however, CCK₂Ri4sv-expressing tumors did not show a significantly increased proliferation rate compared with tumors without the splice variant, although the number of investigated cases may be too small to permit definite conclusions. At present, it remains unclear in how far the functional effects of CCK₂Ri4sv observed in vitro may be extrapolated to original tumors. It has in particular to be considered that the CCK₂Ri4sv expression in its natural environment may show significant differences to that in experimental models. Indeed, transfected cell lines used to explore CCK₂Ri4sv functionality express the splice variant abundantly and selectively [6,7,35], while original tumor tissues express CCK₂Ri4sv transcripts at very low levels and always together with high levels of wild-type CCK₂ receptor and sometimes even CCK₁ receptors [2]. In vivo, the CCK₂Ri4sv may well dimerize with either of these receptors, similar to the wild-type CCK₂ receptor [36]. Would such heterodimers behave in terms of receptor binding and signaling more like wild-type CCK₂ or CCK₁ receptors than like the CCK₂Ri4sv, or would they elicit even new characteristics? Moreover, as CCK₂Ri4sv is expressed at very low levels in original tumors, it may not contribute to cell proliferation to the same extent as in the cell line models. Finally, the constitutive activity of the CCK₂Ri4sv, which is largely responsible for the

increased proliferation, may not be present in all original tumors. In fact, it was found to be restricted to certain cell types and to be in particular absent in epithelial tumor cells [7,9]. A possible strategy to further elucidate CCK₂Ri4sv-mediated biologic effects may be to study the association between CCK₂Ri4sv expression and proliferative index or activation of down-stream signaling pathways in an extended number of original tumors. Unfortunately, the use of cancer cell lines as an alternative to transfected cell lines for similar studies is limited, since cancer cell lines exhibit splicing patterns that are significantly different from those occurring in vivo and have often lost splice variant expression [9,15,18,37] (personal observations).

Given its high specificity for and high incidence in clinically important tumors like SCLC and GIST, the CCK₂Ri4sv may represent an attractive target for potential clinical applications [13,14]. The low tumoral CCK₂Ri4sv expression levels imply that only diagnostic tests with high sensitivity, such as PCR, may be clinically useful. Indeed, it has been demonstrated with several examples that PCR for splice variants performed for instance on pleural or peritoneal fluid can assist in tumor diagnosis, despite the very high sensitivity of the method and not exclusive expression of splice variants in cancer cells [38–40]. Conversely, targeting CCK₂Ri4sv with a radioactive ligand, as suggested recently [41], may be a less promising approach because accumulation of sufficient radioactivity within the tumor area for imaging or therapy may not be achieved due to the low tumoral CCK₂Ri4sv expression levels.

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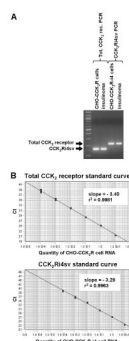


Figure 1.

Real-time PCR: specificities (A) and dynamic ranges (B) of total CCK₂ receptor and CCK₂Ri4sv assays. A: Visualization of real-time PCR products obtained for total CCK₂ receptor and CCK₂Ri4sv in CHO-CCK₂R and CHO-CCK₂R-i4 cells, respectively, as well as in an insulinoma expressing both wild-type and splice variant CCK₂ receptor in an agarose gel. A 100bp DNA ladder was included as molecular weight marker. Both PCR assays yield a single product migrating with the expected size. B: Standard curves of total CCK₂ receptor and CCK₂Ri4sv real-time PCR reactions. Both curves show linearity over a 6-log RNA concentration range.

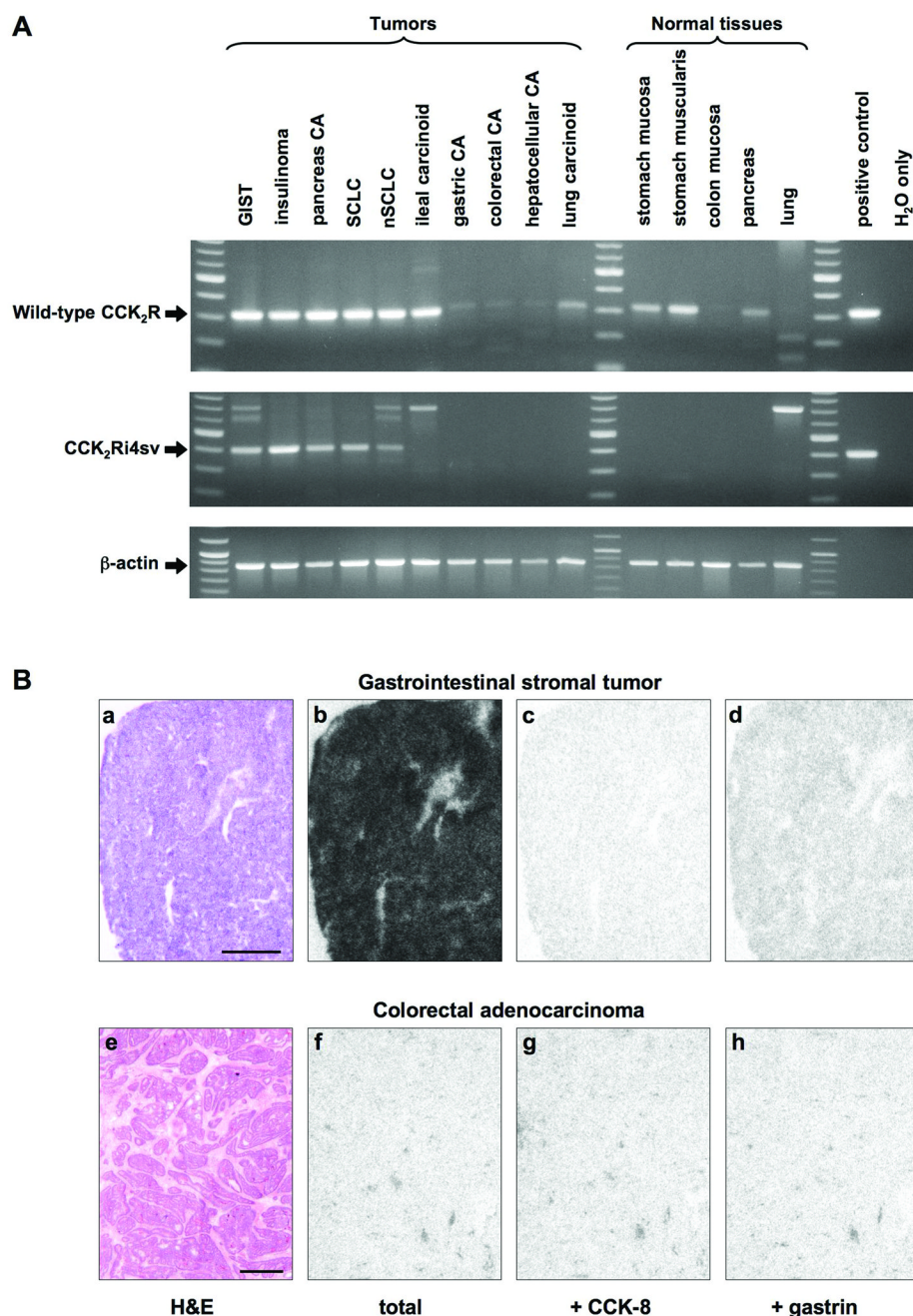


Figure 2.

Expression of CCK₂ receptor transcripts (A) and binding sites (B) in tissues. A: Agarose gels showing representative end-point RT-PCR products for wild-type CCK₂ receptor (top gel), CCK₂Ri4sv (middle gel), and β-actin (bottom gel) in tumors and normal tissues. Positive control reactions were performed on cDNA of CHO-CCK₂R and CHO-CCK₂R-i4 cells for the wild-type and splice variant CCK₂ receptor, respectively. For negative control no template was added (H₂O only). A 100bp DNA ladder served as molecular weight marker. Wild-type CCK₂ receptor transcripts are amplified in all displayed tumors and normal tissues except for normal lung. Note the weak bands obtained in gastric, colorectal, and hepatocellular carcinomas. In the colon mucosa, the band is barely visible. CCK₂Ri4sv

transcripts are detected in the GIST, insulinoma, pancreatic adenocarcinoma, SCLC, and nSCLC, but not in the remaining tumors or normal tissues. Additional bands migrating with higher molecular weights correspond to amplified genomic CCK₂ receptor or are non-specific, as confirmed by sequencing. B: In vitro receptor autoradiography on serial tissue sections to assess CCK receptor binding sites in a GIST (a–d) and a colorectal adenocarcinoma (e–h). a, e: H&E-stained tissue sections showing the tumor tissues (bars = 1mm). b, f: Autoradiograms showing total ¹²⁵I-CCK binding to the tumor tissues. Very strong ¹²⁵I-CCK binding to the GIST, but hardly any labeling of the colorectal carcinoma. c, g: Autoradiograms showing ¹²⁵I-CCK binding in the presence of 50 nM cold CCK-8; and e, h: autoradiograms showing ¹²⁵I-CCK binding in the presence of 50 nM cold gastrin. In the GIST, ¹²⁵I-CCK is completely displaced by CCK-8 and almost to the same extent by gastrin, proving that the vast majority of the ¹²⁵I-CCK binding sites correspond to CCK₂ receptors. (The small fraction of total ¹²⁵I-CCK binding that is displaced by CCK-8 but not gastrin corresponds to concomitantly expressed CCK₁ receptors.) Conversely, in the colorectal carcinoma, the traces of ¹²⁵I-CCK binding are not displaced by either CCK-8 or gastrin, proving that they reflect non-specific ¹²⁵I-CCK binding to the tissue. This tumor has no detectable CCK receptor binding sites.

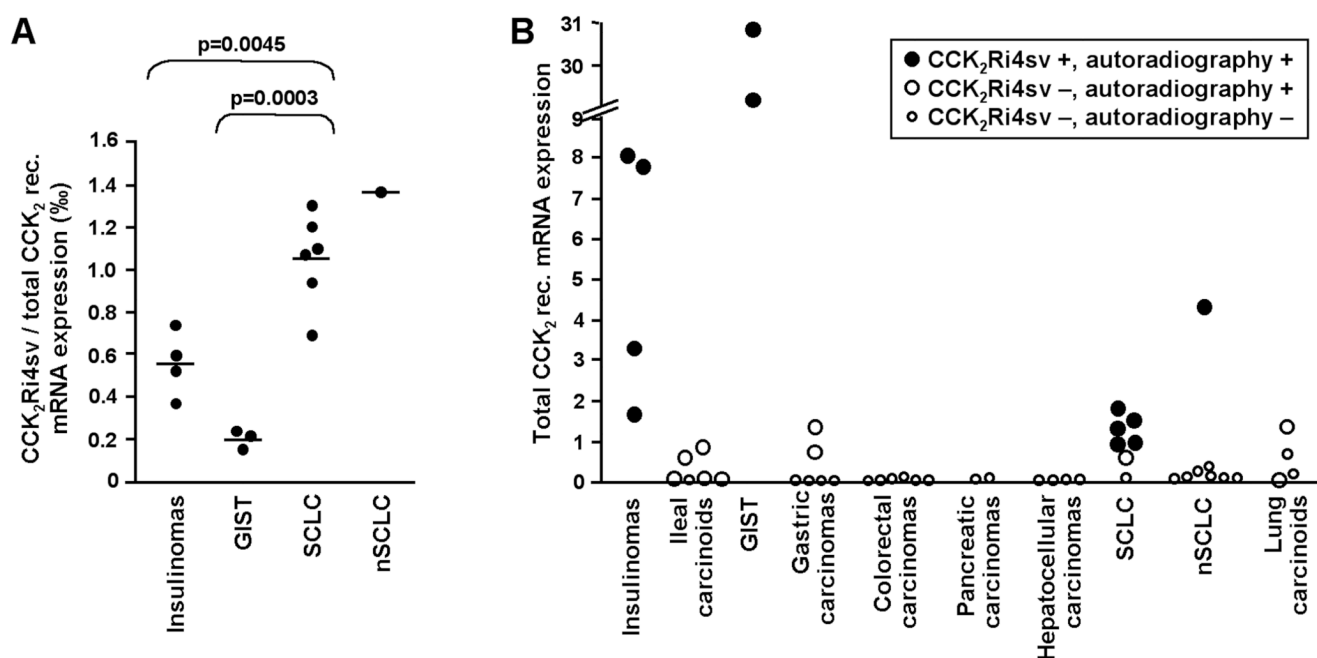


Figure 3.

CCK₂Ri4sv transcript expression in tumors. A: Real-time PCR quantification of CCK₂Ri4sv transcripts relative to total CCK₂ receptor transcripts. Each point represents an individual tumor; scale bars = mean values. In all tumors, the CCK₂Ri4sv transcripts account for a small fraction in the per mill range of total CCK₂ receptor transcripts. SCLC and nSCLC express significantly higher relative amounts of CCK₂Ri4sv transcripts than insulinomas or GIST. B: Total CCK₂ receptor transcripts levels relative to 18S rRNA levels in tumors with CCK₂Ri4sv expression (black points) and without CCK₂Ri4sv expression (white points), as well as in autoradiographically positive tumors (large points) and autoradiographically negative tumors (small points). Total CCK₂ receptors could not be quantified in a GIST and a SCLC because 18S rRNA levels strongly deviated from expected values in the former and not enough RNA was available for endogenous control gene PCR in the latter. The CCK₂Ri4sv is identified only in tumors with relative total CCK₂ receptor levels above 1, whereas almost all tumors without CCK₂Ri4sv expression show total receptor transcript levels below 1. Furthermore, most of the tumors without autoradiographically measurable CCK₂ receptor binding sites exhibit very low total receptor transcript levels, whereas the tumors with identifiable receptor binding sites express higher transcript levels.

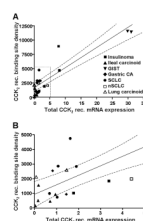


Figure 4.

Correlation of the CCK₂ receptor binding site density measured with autoradiography with receptor transcript levels relative to 18S rRNA levels assessed by real-time PCR in autoradiographically positive tumors. B is a higher magnification of the area indicated with dotted lines in A. The straight line represents the line of best fit obtained by linear regression analysis (dashed lines = 95% confidence intervals).

Primers and TaqMan MGB probe used for end-point and real-time PCR: sequences, complementary gene regions, and amplicon sizes

Table 1

Target	Primer / probe	Sequence (5'-3')	Gene region	Amplicon size
End-point PCR	Total CCK ₂ receptor *	Sense primer AATCGCAGCGTGAGCAGGTG	Exon 1	2032 nt ^{††}
		Antisense primer AGCAATGGAGGGAGTGGGAG	Exon 5	
	Wild-type CCK ₂ receptor [†]	Sense primer CGCGTGATTGTAGCCACGTG	Exon 3	311 nt
		Antisense primer TTCTGGTGAACAGCCCTCTGG	Junction exons 4/5	
	CCK ₂ Ri4sv [†]	Sense primer CTCGCGTGATTGTAGCCAC	Exon 3	399 nt
Real-time PCR		Antisense primer CTTCCTTCTCACCCCTCAC	Intron 4	
	β-actin	Sense primer CCAGCTCACCATTGGATGATATCG	Junction exons 1/2	853 nt
		Antisense primer GGAGTTGAAGGTAGTTTCGTGGATGC	Exon 5	
	Total CCK ₂ receptor	Sense primer CGCCAGACCTGGTCCGTACT	Junction exons 3/4	164 nt
		Antisense primer GCCCGCCTTGGTTTCG	Exon 4	
Real-time PCR		Probe 6FAM-TCGCTGTCACCTGTCGC	Exon 4	
	CCK ₂ Ri4sv	Sense primer CGCCAGACCTGGTCCGTACT	Junction exons 3/4	227 nt
		Antisense primer CCCGTCGCCCTCCAAA	Intron 4	
		Probe 6FAM-TCGCTGTCACCTGTCGC	Exon 4	

* First round of nested PCR
[†] second round of nested PCR
^{††} nt = nucleotides

For reference sequences, see GenBank accession no. NM_176875 (human CCK₂ receptor) and NG_007992 (human β-actin)

Expression of wild-type CCK₂ receptor and CCK₂Ri4sv transcripts and total CCK₂R binding sites in the same gastrointestinal and lung tumor tissues

Table 2

	Wild-type CCK ₂ receptor transcripts		CCK ₂ Ri4sv transcripts		Total CCK ₂ receptor binding sites	
	Incidence: Positive / total cases (%)	Incidence: Positive / total cases (%)	Incidence: Positive / total cases (%)	CCK ₂ Ri4sv / total CCK ₂ receptor transcripts: Mean ± SEM	Incidence: Positive / total cases (%)	Density: Mean ± SEM (dpm/mg)
Insulinomas	4 / 4 (100%)	4 / 4 (100%)	4 / 4 (100%)	0.56 ± 0.08 ‰	4 / 4 (100%)	4076 ± 1760
Ileal carcinoid tumors	8 / 8 (100%)	0 / 8 (0%)	0 / 8 (0%)		7 / 8 (88%)	629.2 ± 217
GIST	4 / 4 (100%)	4 / 4 (100%)	4 / 4 (100%)	0.21 ± 0.02 ‰	4 / 4 (100%)	6590.5 ± 545
Gastric adenocarcinomas	6 / 8 (75%)	0 / 8 (0%)	0 / 8 (0%)		2 / 8 (25%)	1745 ± 806
Colorectal adenocarcinomas	9 / 13 (69%)	0 / 13 (0%)	0 / 13 (0%)		0 / 13 (0%)	
Pancreatic adenocarcinomas	4 / 7 (57%)	1 / 7 (14%)	1 / 7 (14%)	NT*	0 / 7 (0%)	
Cholangiocarcinomas	0 / 3 (0%)	0 / 3 (0%)	0 / 3 (0%)		0 / 1 (0%)	
Hepatocellular carcinomas	4 / 5 (80%)	0 / 5 (0%)	0 / 5 (0%)		0 / 5 (0%)	
SCLC	8 / 9 (89%)	6 / 9 (67%)	6 / 9 (67%)	1.05 ± 0.09 ‰	7 / 9 (78%)	2054.8 ± 702
nSCLC	9 / 12 (75%)	1 / 12 (8%)	1 / 12 (8%)	1.37 ‰	1 / 12 (8%)	1975
Lung carcinoid tumors	7 / 8 (88%)	0 / 8 (0%)	0 / 8 (0%)		4 / 8 (50%)	1272 ± 635

* NT = not tested due to limited availability of sample